

ON SPERMATOGENESIS, NUMBERS OF SERTOLI CELLS AND LEYDIG CELLS IN STALLIONS AS MODULATED BY AGE AND SEASON

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INTRODUCTION

Spermatogenesis has known to be affected in a variety of ways when the testis is subjected to different physiological treatments. A quantitative histological analysis of testicular cells is therefore necessary to evaluate the extent of the changes in spermatogenesis (Lino, 1971). Extensive studies on the influence of age and season on the reproductive capacity of the stallion are available in the literature (See Johnson & Thompson, 1983; Johnson & Tatum, 1989). However, the consequences of seasonal and age-related changes in numbers of germ cells, Sertoli cells and Leydig cells have received limited attention. Since stallion experience several seasonal and age-related influences, horse testis may serve as a useful model for studying the corresponding possible changes in numbers of these cell populations (Johnson & Tatum, 1989).

Therefore, the present investiga-

tion was intended to: 1) determine the changes in numbers of germ cells, Sertoli cells and Leydig cells as modulated by age and season; and, 2) determine if a relationship existed between the number of either of the two somatic testicular cells and spermatogenesis in Arab and native stallions.

MATERIAL AND METHODS

General:

Testes were collected from 28 Arab and native stallions with unknown breeding history along a complete annual cycle. These animals were surgically castrated at the Department of Surgery, Faculty of Veterinary Medicine, Giza and the Veterinary Hospital of the Armed Forces. All stallions were clinically sound and their testes were normal both grossly and microscopically. Tooth replacement and wear were used to determine the age of horses which ranged

form 3-18 years.

Age related data were assigned to three groups : group I (3-<6 years, n=12) , group II (6-<13 years , n=26) and group III (13-<18 Years , n=18 testes). The four seasonal periods were designated as : winter (December - February , n=16) , spring (March - May , n=14) , summer (June - August , n=14) and autumn (September - November , n=12 testes). The testes from each horse were weighed immediatly upon removal. The tunica albuginea was removed and weighed , and the weight of testicular parenchyma was calculated as the difference . Left and right testes from each stallion were used for quantitative determinations of germ cells , Sertoli cells and Leydig cells .

Histological evaluations :

For these evaluations , small pieces of testicular tissue were fixed in Bouin's solution , dehydrated in ascending grades of ethanol , cleared in xylene , embedded in paraffin and sectioned at 5-7 μ thick . Sections were stained with periodic acid-Schiff's reagent (PAS) and counterstained with hematoxylin (Drury & Wallington , 1980) . Two slides per testis , each containing sections of tissues from the same tissue block but separated by several mm , were prepared . Spermatogenesis was assessed by determining the number of spermatogonia, young and old primary spermatocytes, round sper-

matids and Sertoli cells nuclei a stage I (Swierstra *et al.* , 1974) c the cycle of the seminiferous epithelium (Berndtson , 1977 Berndtson *et al.* , 1983). Analyse of the germ cells and Sertoli cell were performed on cross-section of the seminiferous tubules, where the number of the different cell type was estimated by counting their distinctive nuclei. These cells were enumerated in a total of 50 round tubular cross- sections for each stallion . Leydig cell number wa determined in a total of 50 random microscopic fields (ocular, 15X objective 40) per stallion. The maximum length and the maximum width of the Sertoli cell nuclei as well as the diameters of Leydig cells and their nuclei were measured using a calibrated ocular micrometer. The resulting crude counts were converted to true counts by Abercrombie's procedure, where true count = crude count x (section thickness) + section thickness + nuclear diameter (measurements in microns, Abercrombie, 1946; Berndtson, 1977)

Statistical Analysis:

Data were expressed as mean \pm s.e.m. The effects of consecutive ages and seasons were tested by one- way analysis of variance. If the F- value was significant, differences in means amongst groups were evaluated by the Studentized range Q method. The relationships amongst the studied parameters

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Table (1) : Effect of age on the studied testicular parameters (mean \pm SEM) .

Parameter	Group I 3 - < 6 yrs (n = 12)	Group II 6 - < 13 yrs (n = 26)	Group III 13 - < 18 yrs (n = 18)	Overall Mean (n = 56)
Parenchymal weight (g)	152.86 ^c \pm 3.74	163.90 ^{ad} \pm 2.98	137.78 ^b \pm 3.93	153.14 \pm 2.57
Numbers of germ cells: ¹				
Spermatogonia	3.28 ^a \pm 0.11	4.08 ^b \pm 0.13	3.20 ^a \pm 0.24	3.62 \pm 0.06
Young primary spermatocytes	13.67 ^a \pm 0.47	17.00 ^b \pm 0.52	13.60 ^a \pm 0.42	15.21 \pm 0.27
Old primary spermatocytes	16.12 ^a \pm 0.60	20.40 ^b \pm 0.63	16.00 ^a \pm 0.50	18.10 \pm 0.36
Round spermatids	54.82 ^a \pm 2.03	68.00 ^b \pm 2.11	53.21 ^a \pm 1.81	60.42 \pm 1.11
Sertoli cells: ¹				
Numbers of cells	15.37 ^c \pm 0.42	16.70 ^{ad} \pm 0.47	13.21 ^b \pm 0.61	15.29 \pm 0.33
Length of nucleus (μ m)	12.01 \pm 0.25	11.65 \pm 0.45	11.85 \pm 0.29	11.79 \pm 0.27
Width of nucleus (μ m)	6.26 \pm 0.09	6.25 \pm 0.12	6.25 \pm 0.10	6.25 \pm 0.07
Maximum nuclear diameter (μ m)	18.27 \pm 0.28	17.90 \pm 0.47	18.10 \pm 0.23	18.00 \pm 0.35
Leydig cells: ²				
Numbers of cells	85.11 ^c \pm 2.38	92.15 ^{ad} \pm 1.95	74.56 ^b \pm 1.80	84.99 \pm 1.78
Diameters of cells (μ m)	14.63 \pm 0.35	14.82 \pm 0.51	15.35 \pm 0.47	14.95 \pm 0.28
Diameters of nuclei (μ m)	6.42 \pm 0.12	6.59 \pm 0.10	6.55 \pm 0.14	6.54 \pm 0.04

1 Numbers per stage I tubular cross-section .

2 Numbers per microscopic field (ocular, 15 x objective 40)

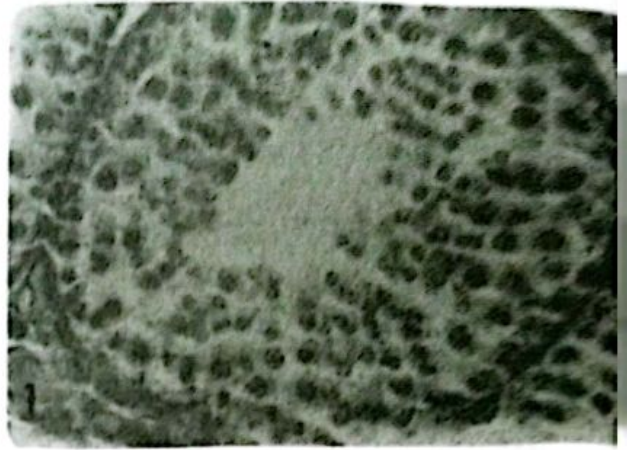
a, b Means in rows with different superscripts differ (P<0.01)

c, d Means in rows with different superscripts differ (P<0.05)

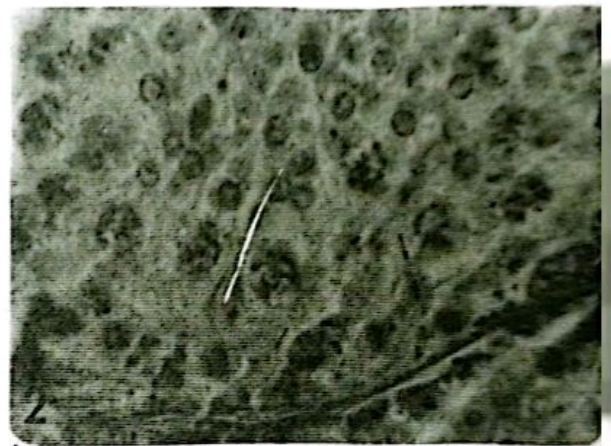
were estimated by correlation coefficients. All statistical methods were carried out according to Snedecor and Cochran (1976).

RESULTS

Histological evaluation of the numbers of spermatogonia, young and old primary spermatocytes, round spermatids and Sertoli cells nuclei per stage I tubular cross-section, revealed that all stallions had active spermatogenesis. stage I of the seminiferous epithelium cycle was identified in tubules from the complete disappearance of luminal spermatozoa to the onset of elongation of spermatid nuclei (Fig.1). The spermatids were usually located in 3 to 4 layers and appeared as round cells with pale nuclei, under them there were two layers of spermatocytes. The upper layer of spermatocytes was older and their nuclei were in pachytene phase. The lower layer on the basal membrane, between the spermatogonia, young spermatocytes (leptotene) were observed. The basement membrane was lined with a few A spermatogonia and their nuclei were pale and contain chromatin in the form of dust-like particles (Fig. 1). The nuclei of Sertoli cells appeared slightly separated from the tubular membrane and had a polymorphous shape with a large nucleolus and a general distribution of chromatin in fairly fine granulations (Figs. 2 & 3). The inte



**Fig.(1): A section of stallion testis in stage I of the seminiferous epithelial cycle.
(PAS technique , X 410)**



**Fig.(2): A section of mature stallion testis in stage I during the breeding season showing the abundant numbers of germ cells and Sertoli cells (arrows).
(PAS technique , X 800)**

stitial tissue appeared as a narrow spaces or triangular areas between the seminiferous tubules. The interstitial or Leydig cells made up the majority of the interstitial tissue and were almost tightly packed together accompanied with vessels. The Leydig cells had a polygonal or round shape with large



Fig.(3): A section of a testis of mature stallion in stage I during the non-breeding season. Notice the decrease in number of germ cells and Sertoli cells (arrow).
(PAS technique, X 800)



Fig.(4): A section of mature stallion testis during the breeding season. Note: the abundant number of Leydig cells.

(PAS technique, X 800)

and or slightly oval nuclei (Figs. 4 & 5).

As no significant differences were found between testicular parameters studied for the right and left testes or between Arab and native horses, the data were tabulated irrespective of testis side and breed. The overall mean values (\pm s.e.m.) of parenchymal weight,



Fig.(5): A section of mature stallion testis during the non-breeding season.

Notice: The decrease in number of Leydig cells.

(PAS technique, X800)

numbers of germ cells, numbers of Sertoli cells and numbers of Leydig cells as well as the dimensions of Sertoli cell nuclei, Leydig cells and Leydig cell nuclei are presented in Table 1.

The pattern of changes in the mean values (\pm s.e.m.) of the studied testicular parameters due to age are depicted in Table 1. Age influenced parenchymal weight ($P < 0.05$), numbers of all germ cell types ($P < 0.01$), numbers of Sertoli cells ($P < 0.01$) and numbers of Leydig cells ($P < 0.01$). The highest values for these criteria were achieved by stallions of age group II (6 - < 13 years), whereas the lowest values were reported later in life. Age had no significant effect of the three dimensions of Sertoli cell nuclei, diameter of Leydig cells and/or diameter of Leydig cell nuclei (Table. 1). Based upon the number of round spermatid "the most advanced germ cells enumer-

Table (2) : Correlation coefficients amongst the studied parameters.

Parameter	Numbers of Sertoli cells	Numbers of Leydig cells	Numbers of Spermatogonia
Age : < 13 Years (n=38)	0.702**	0.717**	0.642**
Overall (n=56)	-0.215	-0.224	-0.180
Parenchymal weight	0.760	0.690**	0.768**
Numbers of Sertoli cells	-----	0.638**	0.600**
Numbers of Leydig cells	0.638**	-----	0.587**
Numbers of germ cells :			
Spermatogonia	0.600**	0.587**	-----
Young primary spermatocytes	0.553**	0.549**	0.860**
Old primary spermatocytes	0.640**	0.590**	0.854**
Round spermatids	0.629**	0.580**	0.840**

** Significant at 1% level

highest ($P < 0.01$) in stallions of age group II. Nevertheless, in group I and group III, the production of spermatozoa averaged only about 80% and 78%, respectively of the sperm production rate for stallions of age group II.

The relationships amongst the studied testicular parameters are listed in Table (2). Age and numbers of each of Sertoli cells, Leydig cells and spermatogonia were highly ($P < 0.01$) correlated up to 13 years old stallions, whereas a reverse relationships were existed later in life. Numbers of spermatogonia, Sertoli cells and Leydig cells accounted for 59% , 58% and 48% of the variation in parenchymal weight, respectively. On the other hand, highly significant ($P < 0.01$) correlations were obtained between numbers of each of germ

cells and numbers of either of Sertoli cells or Leydig cells (Table 2).

Seasonal changes in the mean values (\pm s.e.m.) of the studied parameters are presented in Table (3). With the exception of the dimensions of Sertoli cell nuclei and diameters of Leydig cells and their nuclei, season exerted a profound effect ($P < 0.01$) on all testicular criteria studied (Table 3). The highest values for parenchymal weight, numbers of each of germ cells, Sertoli cells and Leydig cells were observed in spring and winter (Figs. 2 & 4), followed by summer, then reaching their lowest values in the autumn (Figs. 3 & 5). Seasonal changes in the number of spermatogonia or spermatocytes ultimately reflected in similar changes in the number of more advanced

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Table (3) : Effect of season on the studied testicular parameters (mean \pm SEM) .

Parameter	Winter (n = 16)	Spring (n = 14)	Summer (n = 14)	Autumn (n = 12)
Age (Years)	9.78 ± 0.93	10.39 ± 1.16	8.96 ± 1.10	10.04 ± 1.22
Parenchymal weight (g)	157.75 ^a ± 3.33	166.10 ^a ± 4.42	145.71 ^b ± 4.39	135.62 ^b ± 5.92
Numbers of gerin cells:¹				
Spermatogonia	3.74 ^a ± 0.10	4.45 ^b ± 0.12	3.11 ^c ± 0.14	3.00 ^c ± 0.10
Young primary spermatocytes	15.46 ^a ± 0.47	20.80 ^b ± 0.80	12.50 ^c ± 0.56	11.45 ^c ± 0.48
Old primary spermatocytes	18.61 ^a ± 0.53	24.25 ^b ± 0.56	14.81 ^c ± 0.70	14.14 ^c ± 0.46
Round spermatids	59.40 ^d ± 3.78	70.24 ^{ae} ± 2.47	56.10 ^b ± 2.34	53.71 ^b ± 2.50
Sertoli cells:¹				
Numbers of cells	15.70 ^d ± 0.55	17.52 ^{ae} ± 0.63	14.13 ^b ± 0.47	13.50 ^b ± 0.58
Length of nucleus (μ m)	11.96 ± 0.25	11.85 ± 0.22	11.68 ± 0.36	11.62 ± 0.41
Width of nucleus (μ m)	6.25 ± 0.10	6.26 ± 0.10	6.25 ± 0.10	6.25 ± 0.12
Maximum nuclear diameter (μ m)	18.21 ± 0.28	18.11 ± 0.25	17.93 ± 0.32	17.87 ± 0.47
Leydig cells :²				
Numbers of cells	98.10 ^a ± 3.77	94.89 ^a ± 3.32	74.59 ^{bd} ± 2.11	68.13 ^e ± 2.96
Diameters of cells (μ m)	15.15 ± 0.55	15.40 ± 0.27	14.85 ± 0.45	14.43 ± 0.32
Diameters of nuclei (μ m)	6.60 ± 0.19	6.62 ± 0.16	6.51 ± 0.21	6.40 ± 0.14

¹ Numbers per stage I tubular cross-section .

² Number per microscopic field (ocular, 15 x , objective 40)

a,b,c Means in rows with different superscripts differ (P<0.01)

d,e Means in rows with different superscripts differ (P<0.05)

germ cells and all displayed similar seasonal changes as did the other testicular criteria studied (Table 3). The sperm production rate during winter, summer and autumn averaged 84%, 80% and 76% of that reported in spring, respectively.

DISCUSSION

On the basis of quantitative histological evaluation of the numbers of germ cells and Sertoli cell nuclei per stage I (Swierstra *et al.*, 1974) seminiferous tubular cross-section, all stallions had active spermatogenesis. However, the present results revealed further evidence on the age-related changes in spermatogenesis, parenchymal weight, numbers of Sertoli cells and numbers of Leydig cells. Age-related increase in sperm production rate up to 13 years old stallions, coincided with a similar increase in parenchymal weight, numbers of Sertoli cells and Leydig cells. These findings are in partial agreement with Johnson & Neaves (1981), Johnson & Thompson (1983, 1987) and Johnson and Tatum (1989), who reported that horses experienced an increase in testicular weight, numbers of Leydig cells and Sertoli cells with advancing age up to 20 years, and the changes in numbers of spermatids largely occurred with corresponding changes in parenchymal weight and numbers of somatic testicular cells. Moreover, a similar peak was reported in the gonadal sperm re-

serve (El-Wishy *et al.*, 1980), the extragonadal sperm reserve (Amann *et al.*, 1979) and the number of ejaculated spermatozoa (Pickett *et al.*, 1979; El-Baghdady *et al.*, 1990). The strong relationships reported herein between parenchymal weight and numbers of each of germ cells, Sertoli cells or Leydig cells support the speculation of Berndtson *et al.* (1987) that testicular development might continue until Sertoli cells had reached their maximal capacity for maintaining the integrity of the blood-testis barrier, providing physical contact with germ cells and providing a biochemical milieu favourable to germ cell development. The increase in number of spermatogonia in stallions of 6-13 year old (group II), is consistent with increased parenchymal weight and numbers of somatic cells including Sertoli cells which might be driven by the need to accommodate more spermatogonial progeny at that age (Berndtson *et al.*, 1987; Johnson & Tatum, 1989). Similarly, number of Sertoli cells has been correlated with number of spermatogonia in the rat, ram and bull (Hochereau-de Reviers & Courrot, 1978). The finding that the germ cell: Sertoli cell ratio increased from young (group I) to mature (group II) or old (group III) stallions (5.72 vs 6.56 vs 6.51, respectively) is confirmed by Johnson & Thompson (1983) and Jones & Berndtson (1986), who reported a predictable increase in

stallions. However, once the maximal germ cell: Sertoli cell ratio has been reached (Johnson & Tatum, 1989), the innate capacity of seminiferous tubules to house only 8% and 21% more Sertoli cells in mature over young and old stallions, respectively limits the total number of germ cells that can be accommodated in the testis. Hence, the number of Sertoli cells is related to parenchymal weight, numbers of spermatogonia and numbers of round spermatids, changes in numbers of Sertoli cells may regulate spermatogenesis in the stallion (Johnson & Tatum, 1989), through support and nutrition of germ cells, spermiation of mature spermatids, movements of young germ cells, phagocytosis of degenerating germ cells and residual bodies, secretion of proteins, formation of blood testis barrier, cell-to-cell communication (Dym & Madhwa Raj, 1977) and through secretion of a mitogenic polypeptide (Feig et al., 1980). Increased values for Leydig cell number in mature stallions (6- < 13 years) is consistent with increased Leydig cell function namely testosterone production (Johnson & Neaves, 1981), which is required for completion of meiosis during spermatogenesis (Steinberger, 1971). The apparent reduction in numbers of Leydig cells reported in older ages (group III) would result in a corresponding decline in testosterone level (Gusmao et al., 1988; Berndtson & Jones, 1989) and

may increase the rate of cell loss during the postprophase division which ultimately result in reduced rate of spermatogenesis (Johnson & Thompson, 1983). Increased Leydig cell numbers have been induced experimentally by HCG administration in adult rats and mitotic figures were seen in the interstitium on rare occasions (Christensen & Peacock, 1980). It is also possible that Leydig cell numbers may have augmented through differentiation of other interstitial cells into recognizable Leydig cells (Johnson & Neaves, 1981). In some postpubertal stallion testes, Johnson & Neaves (1981) observed groups of cells that appeared to be transition between fibrocytes and definitive Leydig cells. However, this assumption cannot be ruled out until a complete histometric census of all interstitial cell types according to age is performed to evaluate this possibility. Evidence from the present study cleared that numbers of Sertoli cells, Leydig cells and germ cells are interrelated. Therefore, these results emphasize the importance of age changes in numbers of Sertoli cells and Leydig cells on regulation of stallion spermatogenesis. Moreover, age alteration in parenchymal weight might be influenced by age changes in numbers of spermatogonia and numbers of somatic testicular cells.

Unlike some classical seasonal

breeders that cease spermatogenesis in the non-breeding season (Short & Mann, 1966; Neaves, 1973), spermatogenesis in the stallion continued at a reduced rate throughout the non-breeding season (June-November). Based upon the number of round spermatids, sperm production rate in the spring was 15% , 20% and 24% higher than winter, summer and autumn, respectively. The current results confirmed the observations of Berndtson *et al.* (1983), Johnson & Thompson (1983) and Johnson & Tatum (1989) on the seasonality of equine spermatogenesis and the nature of spermatozoa production by a quantitative histological technique. Seasonal changes in spermatogenesis may be a function of germ cell degeneration during meiosis and seasonal modulation of the number of A spermatogonia (Johnson, 1991). In agreement with Johnson & Thompson (1983) and Johnson (1985), seasonal changes in numbers of both Sertoli and Leydig cells largely occurred with corresponding changes in parenchymal weight; changes in sperm production rate is consistent with seasonal changes in parenchymal weight and number of Sertoli cells. Also, the increase in number of spermatogonia is consistent with increased parenchymal weight and numbers of somatic testicular cells (Johnson & Tatum, 1989). These seasonal changes could be induced by photoperiod, which drives seasonal changes in serum concentra-

tions of LH, FSH and testosterone and/or some unknown factors (Clay *et al.*, 1988; Johnson & Tatum, 1989). Therefore, spring peak in parenchymal weight and sperm production rate, could be explained by an elevated A spermatogonia (Johnson, 1985, Johnson & Tatum, 1989; Johnson 1991), the numbers of Sertoli cells (Johnson & Nguyen, 1986) and Leydig cells (Johnson & Thompson, 1986; 1987).

While the source of additional Sertoli cells in the breeding season (spring and winter) and their fate after the breeding season are unknown, Johnson & Nguyen (1986) claimed that interstitial growth along the length of the seminiferous tubules (such as would be produced by mitotic activity) is how the Sertoli cell population is augmented in the breeding season. The lack of age and seasonal effects on the average diameter of Sertoli cell nuclei was confirmed by average maximum nuclear diameter (height and width). This finding of similar nuclear size agrees with those found in light horse breed (Johnson & Nguyen 1986) or other seasonal breeders such as the red deer (Hocheureau de Reivers & Lincoln, 1978). Moreover, similarity in size of Sertoli cell nucleus amongst seasons and age groups, may reflect a continued base-line functional state of Sertoli cells in all ages studied throughout the year in which sperm

production continues (Thompson et al., 1977; Johnson & Nguyen, 1986). Therefore, the number of Sertoli cells rather than their nuclear size may regulate spermatogenesis in the stallion. Increased values for Leydig cell number in spring and winter (26% higher) compared with summer and autumn is consistent with increased Leydig cell function namely testosterone production. Previous studies of circulating testosterone in horses have shown higher levels in the breeding season (Berndtson et al., 1974); these higher levels were significantly correlated with intratesticular testosterone contents (Berndtson et al., 1983; Johnson & Thompson, 1986, 1987; Berndtson & Jones, 1989). Both circulating and intratesticular testosterone levels may be modulated by seasonal fluctuations in concentrations of LH (Thompson et al., 1977), which influenced Leydig cell numbers (Christensen & Peacock, 1980; Johnson & Thompson, 1983, 1987). Similarly, Johnson and Thompson (1987) reported that the number of Leydig cells per testis was 53% greater in the breeding season than the non-breeding season and the intratesticular testosterone content was significantly related to the number of Leydig cells. The present results emphasize the importance of seasonal changes in numbers of Leydig cells on the amount of smooth endoplasmic reticulum (SER) available to produce

testosterone and on testosterone content per testis in the stallion. Furthermore, the effect of season on testicular steroidogenesis in stallions may be a consequence of changes in numbers of Leydig cell rather than steroidogenic capacity of individual Leydig cell (Johnson & Thompson, 1987; Clay et al., 1988). Lack of seasonal influences on the size or cytoplasmic composition of individual Leydig cell was previously noted by Johnson & Thompson (1987) and was attributed to the continued production of sperm in the stallion throughout the year. In contrast, other seasonal breeders such as the rockhyrax (Neaves, 1973) or Soay ram (Houchereau-de Reivers et al., 1985), the seasonal effect on testosterone production was related to the size of Leydig cells rather than their absolute numbers. Both strategies result in a change in the amount of SER per testis, which appear to be the primary determinant of steroidogenic capacity (Zirkin et al., 1980; Johnson & Thompson, 1986, 1987).

In conclusion, the numbers of both Sertoli cells and Leydig cells fluctuate with the yearly reproductive cycle of the stallion resulting in their peak values in mature stallions (6 - < 13 years old) and in the spring and winter seasons. Moreover, age and seasonal changes in numbers of both somatic testicular cells largely occurred with a corresponding change in parenchymal

weight and spermatogenesis in Arab and native stallions.

SUMMARY

The study used 56 testes collected from 28 Arab and native stallions (3-18 years) during a complete annual cycle. The consequences of seasonal and age-related changes in parenchymal weight and in numbers of spermatogonia, young and old primary spermatocytes, round spermatids, Sertoli cells and Leydig cells were evaluated. There were statistically significant seasonal and age effects on parenchymal weight, numbers of Sertoli cells, Leydig cells and spermatogenesis. Seasonal changes in the above testicular parameters were maximal in spring and winter, followed by summer, then reaching their minima in the autumn. The highest values for these criteria were reached by stallions of 6- <13 years, whereas the lowest values were observed later in life. Neither age nor season influenced the diameter of Sertoli cell nuclei, Leydig cell or Leydig cell nuclei. The mechanisms by which the numbers of both somatic testicular cells fluctuate with the yearly reproductive cycle of the stallion were discussed. Evidence from the present results revealed that numbers of Sertoli cells, germ cells and parenchymal weight were interrelated. Numbers of spermatogonia, Sertoli cells and Leydig cells accounted for 59% 58% and 48% of the variation in parenchymal weight, respectively. The relationships existed between the number of either of the two somatic testicular cells and spermatogenesis were also scrutinized. The present results emphasize the importance of age and seasonal changes in numbers of Sertoli cells and Leydig cells on regulation of stallions spermatogenesis.

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